# Development of a Quantitative Cell Culture-based Infectivity Assay for *Cryptosporidium parvum*

## Project Scope

Oocysts of the enteric protozoa *Cryptosporidium parvum* are ubiquitous in surface waters; have a low infectious dose, even in healthy individuals; and are resistant to conventional water treatment practices. For these reasons, documented waterborne outbreaks of cryptosporidiosis associated with contaminated drinking water or recreational water occur every year in the United States. Therefore, waterborne *C. parvum* oocysts have been of steadily increasing public health and regulatory concern in the past decade. For example, improved control of *C. parvum* is a key component in the proposed Long-Term 2 Enhanced Surface Water Treatment Rule.

At the time the research conducted under this grant was being planned, important limitations of the widely used immunofluorescent assay (IFA) for *Cryptosporidium* detection had been identified. One major limitation was their inability to discriminate between *C. parvum* (the only species known to cause disease in humans) and other nonpathogenic species (e.g., *C. muris*). In addition, no routine assays were available to determine whether *C. parvum* oocysts detected in environmental samples were both viable and capable of causing infection. Because of the costs and impracticality of water utilities and commercial laboratories conducting more traditional measures of pathogen viability/infectivity (i.e., human volunteer studies and animal infectivity models), EPA identified the development of cell culture-based infectivity assays for *C. parvum* as a pressing drinking water research need. The overall goal of this grant was to develop a quantitative cell culture infectivity assay in combination with a rapid molecular screening assay for *C. parvum* in both finished drinking water and source waters. The specific project objectives included:

### Grant Title and Principal Investigators

Development of a Quantitative Cell Culture-Based Infectivity Assay for *Cryptosporidum parvum* in Source and Finished Water (EPA Grant #R825146)

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### Key Findings and Implications

Analytical Accomplishments:

- Demonstrated that infectious *C. parvum* can be detected by reverse transcriptase-PCR amplification of heat shock protein messenger RNA expressed in the infected cells.
- Developed a sensitive and specific colorimetric *in situ* hybridization method to detect *C. parvum* infection in cell monolayers. The method uses *C. parvum*-specific probes for direct quantitation of infectious foci and is amenable for routine use while providing a robust assessment of relative level of *C. parvum* infectivity.
- Developed sample clean-up procedures based on immunomagnetic separation which yield samples compatible with PCR detection and cell culture infectivity techniques. The method was used successfully to evaluate the efficiency of ultraviolet and ozone disinfection of *C. parvum*.
- Confirmed that *C. parvum* oocysts recovered by EPA Methods 1622/1623 retained their infectivity.

Implications of Research and Impacts of Results:

- *In vitro* cell culture with molecular based detection methods can be considered as a practical technique for measuring the infectivity of waterborne *C. parvum*.
- The method could lead to similar infectivity assays for other waterborne pathogens, especially emerging pathogens for which current detection methods do not provide information on infectivity.

Publications include 7 peer reviewed journal articles and 4 conference/workshop presentations.

**Project Period: October 1996 to September 1998**

## Relevance to ORD's *Drinking Water Research Multi-Year Plan* (2003 Edition)

This project contributes to two of three Long-term Goals for drinking water research: (1) By 2010, develop scientifically sound data and approaches to assess and manage risks to human health posed by exposure to regulated waterborne pathogens and chemicals, including those addressed by the Arsenic, M/MDP, and Six-Year Review Rules; and (3) By 2009, provide data, tools and technologies to support management decisions by the Office of Water, state, local authorities and utilities to protect source water and the quality of water in the distribution system.

The results of this research have provided a sensitive, specific, and quantitative method for determining the infectivity of *C. parvum* oocysts. This enteric protozoan parasite is a well known waterborne pathogen of public health and regulatory concern and its improved control is a key component in the proposed Long-Term 2 Enhanced Surface Water Treatment Rule. The assay provides a better method to evaluate the efficacy of disinfectants, including ultraviolet light and ozone. The method may also be used to monitor oocyst presence and survival in water over time and has been shown to be a practical and inexpensive alternative to animal infectivity assays. In this regard, *in-vitro* cell culture is gaining widespread acceptance for assessing infectivity of waterborne *C. parvum* and similar methods are being increasingly adopted both nationally and internationally.

1. Optimize a cell culture assay for *C. parvum* that could characterize infectivity using small numbers of oocysts;
2. Develop a molecular detection assay which targets messenger RNA (mRNA) from a *C. parvum* specific heat shock protein;
3. Develop a quantitative *in situ* nucleic acid-based assay for detecting infectious *C. parvum*;
4. Develop sample clean-up procedures compatible with cell culture methods that can recover infectious oocysts; and
5. Evaluate and validate the developed methods on environmental samples.

To carry out this research, the investigators designed, used, and field-tested an innovative approach that combined measuring infectivity by cell culture with the rapidity and specificity of molecular techniques (see Figure 1). The molecular step also served as a confirmatory assay for identifying *C. parvum*. Because of the large amount of research conducted under this grant, the investigators' body of work is summarized below in terms of its major studies, results, and implications.

## Project Results and Implications

Optimization of Cell Culture for Detection of Low Levels of Infectious Oocysts: In order to develop an efficient assay, infection conditions as well as assay methods had to be optimized simultaneously. This research focused on identifying the best combinations of cell line, plating characteristics, cell line growth conditions, and infection conditions for quantitative detection of less than 10 infectious oocysts. To this end, the investigators initially considered three established cell lines, Caco-2, HCT-8, and MDBK cells, on the basis of published literature indicating their susceptibility to *C. parvum* infection, preliminary test results, ease of handling, as well as receptivity to other enteric and waterborne pathogens. The Caco-2 cell line was initially selected because it can support *C. parvum* infection as well as a wide range of enteric pathogens including protozoa, viruses, and bacteria. A series of experiments was then conducted to optimize (e.g., pH for growth) use of the Caco-2 cell line (e.g., development of subclones) for primary use throughout the rest of the project. Later in the project, the HCT-8 cell line was determined to be more sensitive and consistent for infectivity assays.

Determination of Infectivity by RT-PCR: The development of a quantitative infectivity assay for *C. parvum* based on *in situ* hybridization required evaluation and optimization of many different analytical methods.

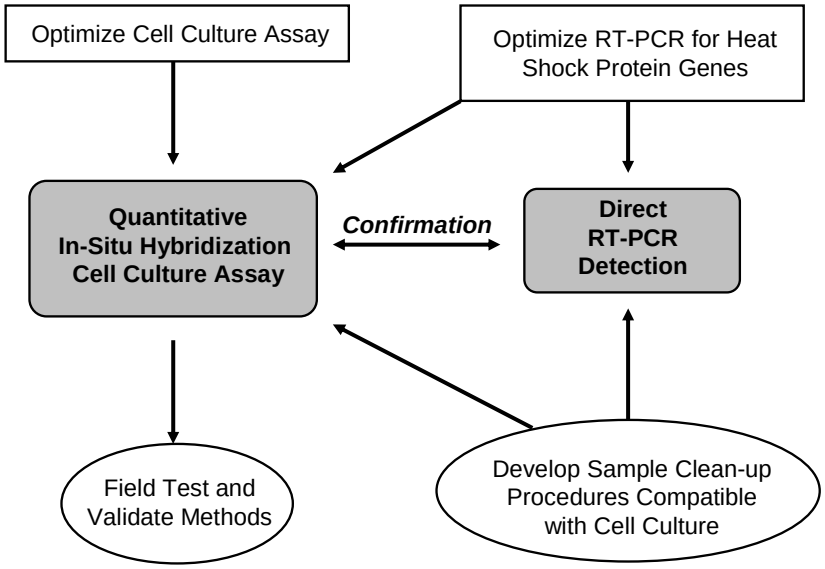


Figure 1. Experimental approach used in the development of a quantitative cell culture infectivity assay in combination with a rapid molecular screening assay for *C. parvum*

Consequently, while developing the infectivity assay, a semi-quantitative method was successfully developed by the investigators to allow optimization of infection conditions, testing different cells lines, and conducting initial sensitivity studies. The latter method was also used for measuring the efficacy of disinfectants for inactivation of *C. parvum*. The procedures consisted of extraction of total RNA from infected cell cultures, purification of mRNA, and detection of *C. parvum*-specific heat shock protein (*hsp70*) mRNA by reverse transcriptase polymerase chain reaction (RT-PCR). In brief, since only oocysts that are viable and infectious will lead to an infection, RT-PCR based detection of mRNA transcribed from a heat shock protein gene provides a specific and sensitive method for detecting infection. In contrast, oocysts that are dead, or viable but non-infectious, will not produce *hsp70* mRNA and thus will not be detected. Amplification products generated by RT-PCR in this research were detected and sized by agarose gel electrophoresis, making it possible to use this approach as a semi-quantitative infectivity assay by digitizing gel images and measuring amplification yields by densitometry.

Development of a Simple and Quantitative Method for Scoring *C. parvum* Infectious Foci: A useful cell culture-based, quantitative infectivity assay for *C. parvum* needs to be species-specific while preserving the ability to detect all stages of parasitic development. The assay must also be easily readable and quantitative. In order to achieve these goals, the investigators had to utilize or develop interim methods to assess progress towards the final goal of a finished detection method. The interim methods assessed were: (1) direct microscopic detection of *C. parvum* stages aided by either Nomarski differential interference contrast (DIC) or Giemsa staining; (2) detection of infectious stages by means of fluorescently labeled anti-sporozoite antibodies; and (3) *in situ*-PCR (IS-PCR) targeting *C. parvum* nucleic acids directly in the infected tissue monolayers. The investigators found that direct microscopic detection of infective oocysts using DIS or Giemsa staining is difficult, time consuming, and often not reliable. Furthermore, fluorescent antibody-based detection of infectious foci was used only as an intermediate method to aid in the development of more specific molecular procedures. In contrast, the investigators found IS-PCR to be an effective alternative quantitative approach for detecting *C. parvum*

infection that combines the easily enumerated reporter molecules used in fluorescent antibody procedures with the sensitivity and specificity of nucleic acid-based PCR techniques. However, IS-PCR was (then) a recently developed technique where simplified protocols and techniques were not commonly available. Thus, it was necessary to develop appropriate experimental positive controls for optimizing each step of the process independently. Ultimately, the investigators concluded that because IS-PCR requires substantial optimization prior to use, the technique may be transferable only to sophisticated laboratories.

An alternative, less complex, and more rapid procedure that the researchers pursued is detection of infectious foci by *in situ* hybridization (ISH). The process of ISH requires target cells to be fixed and permeabilized, suitable probe(s) (in this study, *C. parvum* specific oligonucleotides or amplicon DNA probes) to bind to its specific target, and subsequent detection of the bound probe. The probes may be labeled with a fluorescent marker, referred to as fluorescent ISH (FISH), or with an enzyme moiety that catalyzes the precipitation of a colored substance in a process referred to as colorimetric ISH (CISH). Infectivity is quantified by enumeration of infectious foci by observation under UV microscopy for FISH or under a visible light microscope for CISH (see Figure 2). Enumeration of foci is most likely to be performed manually but could be performed automatically if the microscope is equipped with an automated stage and image analysis software. Using this procedure, clusters of *C. parvum* developmental stage cells were stained dark purple/black and were successfully distinguished from the colorless background of host cells. The CISH protocol for detecting *C. parvum* infection in cell monolayers constitutes the primary contribution of the research conducted under this grant and was evaluated versus more conventional infectivity assays and on environmental samples (see below).

Disinfection Efficacy Studies: This research evaluated the utility of cell culture-based infectivity assays for measuring inactivation of *C. parvum* by ultraviolet (UV) and ozone disinfection. One of the primary applications of an infectivity assay for the water industry is to determine the efficacy of disinfectants and treatment strategies. Because *C. parvum* oocysts are relatively resistant to chlorine disinfection, alternative disinfectants such as UV and ozone have been evaluated and are being increasingly used throughout the United States. To evaluate *C. parvum* inactivation, oocysts were exposed to pulsed UV irradiation and inoculated onto monolayers of HCT-8 cells. After incubation, infectivity was assessed by mRNA extraction and RT-PCR targeting a *C. parvum hsp*70 gene using methods summarized previously. The results demonstrated that up to 3-logs of inactivation (99.9 percent oocyct removal) was achieved with 6.6 $mJ/cm^2$ of UV irradiation. Notably, this inactivation is much greater than some previous studies have suggested. A preliminary comparison between *C. parvum* infectivity in mice (see more below) and cell culture demonstrated good correlation with agreement between the two methods in 83 percent of samples exposed to UV irradiation. For this comparison, 1,000 to 100,000 UV-exposed oocysts were inoculated into CD-1 mice and onto HCT-8 cell monolayers. Infection in cell culture was detected by RT-PCR and the results of both assays were expressed as presence/absence of infection. There was 100 percent correlation between the two assays for control oocysts at a concentration of 50 to 200 oocysts not exposed to UV. Oocysts were also exposed to ozone and then inoculated onto monolayers of HCT-8 cells. After incubation, infectivity was again assessed by mRNA extraction and RT-PCR targeting a *C. parvum hsp70* gene. The results demonstrated a clear dose-response for ozone inactivation with a CT (contact time; a measure of microbe inactivation due to time and concentration of a disinfectant) of 8.4 mg-min/L leading to greater than 99.99 percent inactivation. The results were also in general agreement with the preceding ozone disinfection of *C. parvum* studies. Thus, the UV and ozone disinfection experiments demonstrated that the cell culture-based infectivity assay was an effective tool for measuring the efficacy of alternative disinfectants for *C. parvum* oocysts. Furthermore, the method showed very good correlation with more traditional animal models for studying inactivation kinetics, but was far less expensive, allowing a greater range of parameters to be investigated.

Comparison of Cell Culture and Mouse Infectivity Assays: In this research, cell culture with both CISH and RT-PCR detection were compared to the CD-1 mouse model (conducted at the University of

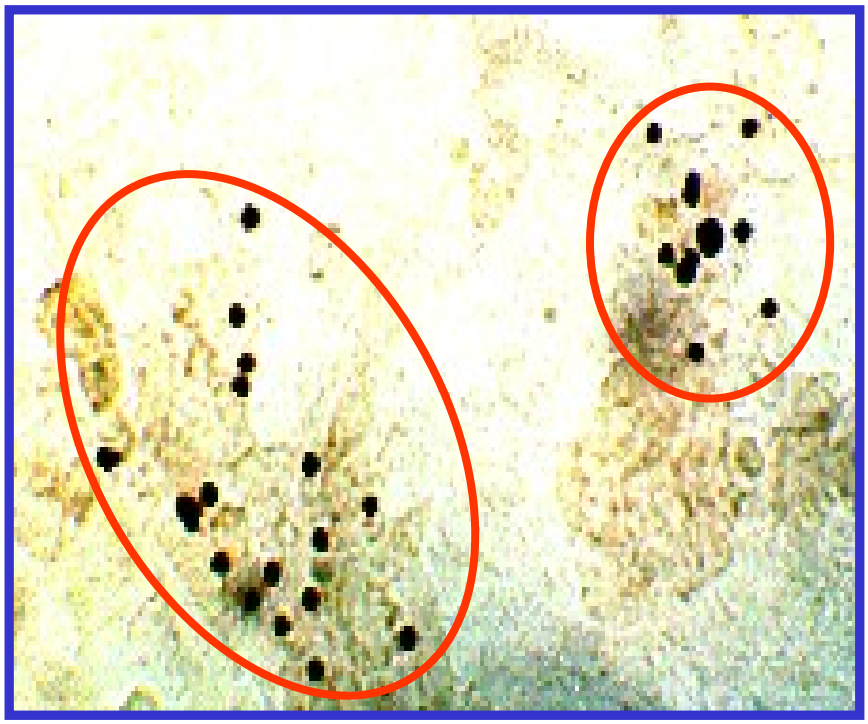

Figure 2. Detection of infectious foci by colorimetric in-situ hybridization (CISH)

Arizona) for measuring infectivity of fresh *C. parvum* oocysts. Measured viability endpoints include *in vitro* excystation and inclusion/exclusion of vital dyes in a variety of mouse models. The widespread acceptance of the mouse infectivity standard is such that they are recognized as the most sensitive and accurate method for measuring infectivity and represent the standard against which all new methods must demonstrate equivalency. Solutions containing 50 to 1,461 oocysts were inoculated in CD-1 mice (through oral delivery of a 10 µL suspension of oocysts) and onto HCT-8 cell monolayers. Infection in CD-1 mice was detected by hematoxylin and eosin staining of intestinal preparations. Using the animal model, the proportion of mice infected ranged from 50 percent with 81 oocysts to 100 percent with 1,461 oocysts, which was found to be consistent with previous animal infectivity studies of *C. parvum*. For oocyst concentrations ranging from 81 to 1,461, there was a significant positive correlation (correlation coefficient = 0.96) between animal infectivity and detection of infection in HCT-8 cells by CISH. The results clearly demonstrate that the cell culture-based infectivity assay with direct quantitative measurement of the level of infection by CISH produced results comparable to the standard mouse infectivity assay. There was also a significant positive correlation between mouse infectivity and infection in HCT-8 cells as detected by RT-PCR (correlation coefficient = 0.85). An additional major advantage of the cell culture-based assay compared to the mouse assay arose from the inability of CD-1 mice to support infection with Genotype 1 *C. parvum* that infects only humans. A high concentration of Genotype 1 oocysts ($10^5$) was inoculated into CD-1 mice but hematoxylin and eosin staining failed to demonstrate evidence of infection. In contrast, Genotype 1 oocysts inoculated onto HCT-8 cell monolayers at concentrations ranging from $10^3$ to $10^5$ did cause infection as measured by RT-PCR.

Development of Environmental Sample "Clean-Up" Procedures that Preserve Infectivity and are Compatible with PCR and Cell Culture Methods: The methods available for concentration and purification of *C. parvum* oocysts from environmental samples at the beginning of this project were inefficient and were not optimized for the specific recovery of infectious oocysts. This research focused on a variety of methods that were developed to "clean-up" environmental water samples prior to or in conjunction with the cell culture infectivity assay that was developed under this grant. The investigators

determined that the conventional purification procedure of gradient centrifugation did not effectively recover oocysts, nor did it provide samples sufficiently free of contaminating or interfering organisms or substances. However, purification methods based on antibody capture, paramagnetic particles, and magnetic concentration—known as immunomagnetic separation (IMS)—had begun to provide an alternative approach to oocyst recovery. Therefore, the researchers conducted experiments in the following areas related to IMS methods for the separation and recovery of *Cryptosporidium* oocysts: paramagnetic bead to oocyst ratios, recovery of oocysts by (IMS- and IFA-based) EPA Methods 1622/1623, suitability of IMS of recovered oocysts for PCR, cell culture infectivity of oocysts following IMS, and cell culture infectivity of oocysts after recovery by EPA Method 1622. Notably, field-testing with seeded environmental water samples showed that oocysts recovered by EPA Methods 1622/1623 retained their infectivity and may be used for source water or finished water monitoring when infectivity information is required.

## Investigators

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## For More Information

## NCER Project Abstract and Reports:

**http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/833/report/0**

## Peer Reviewed Publications

Rochelle, P.A., Ferguson, D.M., Handojo, T.J., De Leon, R., Stewart, M.H., and Wolfe, R.L. 1996. Development of a rapid procedure for *Cryptosporidium*, using in vitro cell culture combined with PCR. Journal of Eukaryotic Microbiology 43:72

Rochelle, P.A., Ferguson, D.M., Handojo, T.J., De Leon, R., Stewart, M.H., and Wolfe, R.L. 1997. An assay combining cell culture with reverse transcriptase-PCR to detect and determine the infectivity of waterborne *Cryptosporidium parvum*. Applied and Environmental Microbiology 63:2029-2037.

Rochelle, P.A., De Leon, R., Johnson, A.M., Stewart, M.H., and Wolfe, R.L. 1999. Evaluation of immunomagnetic separation for recovery of infectious *Cryptosporidium parvum* oocysts from environmental samples. Applied and Environmental Microbiology 65:841-845.

Rochelle, P.A., De Leon, R., Stewart, M.H., and Wolfe, R.L. 1999. Detection, viability and infectivity of waterborne *Cryptosporidium*. Recent Research Developments in Microbiology 3:41-54.

Rochelle, P.A., Ferguson, D.M., Johnson, A.M., and De Leon, R. 2001. Quantitation of *Cryptosporidium parvum* infection in cell culture using a colorimetric in situ hybridization assay. Journal of Eukaryotic Microbiology 48:565-574.

Mofidi, A. A., Baribeau, H., Rochelle, P.A., De Leon, R., Coffey, B.M., and Green, J.F. 2001. Disinfection of *Cryptosporidium parvum* with polychromatic UV light. Journal of the American Water Works Association 93:95-109.

Rochelle, P. A., Marshall, M.M., Mead, J.R., Johnson, A.M., Korick, D.G., Rosen, J.S., and De Leon, R. 2002. Comparison on In Vitro cell culture and a mouse assay for measuring infectivity of *Cryptosporidium parvum*. Applied and Environmental Microbiology 68:3809-3817.